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ABSTRACT

Knowledge on spatio-temporal dynamics of plant primary and secondary chemistry under natural conditions is important to assess how plant defence varies in real field conditions. Plant primary and secondary chemistry is known to vary with both season and vegetation successional stage, however, in few studies these two sources of variation have been examined in combination. Here we examine variations in primary and secondary chemistry of *Jacobaea vulgaris* (Asteraceae) throughout the growing season in early, mid, and late stages of secondary succession following land abandonment using a well-established chronosequence in The Netherlands.

We investigated primary and secondary chemistry of both leaves and flowers, in order to determine if patterns during seasonal (phenological) development may differ among successional stages.

The chemical concentration of primary and secondary chemistry compounds in *J. vulgaris* varied throughout the season and was affected by vegetation succession stage. Concentrations of pyrrolizidine alkaloid (PA) tertiary-amines were highest in flowers during early Summer and in fields that had been abandoned ten to twenty years ago. PA N-oxide concentrations of both leaves and flowers, on the other hand increased with the progression of both season and succession. In Spring and early Summer chlorophyll concentrations were highest, especially in the oldest fields of the chronosequence. During phenological development, nitrogen concentration increased in flowers and decreased in leaves revealing allocation of nutrients from vegetative to reproductive plant parts throughout the growing season.

The highest concentrations of N-oxides and chlorophylls were detected in older fields. Thus, our results suggest that variations in plant patterns of nutritional and defence compounds throughout the growing season are depending on successional context.

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Introduction

Numerous studies have shown variation in primary and secondary chemistry within plants (e.g. Hartmann and Zimmer, 1986; Pichersky and Gang, 2000; Zangerl and Bazzaz, 1992) and that chemistry values may change with plant development stages (e.g. Iason et al., 2012; Walters, 2011) and environments (e.g. Gols et al., 2008; Pyšek et al., 2005). However, very few studies have related variations in plant chemistry during plant development to successional stage, whereas it has been well established that plant population structure can change substantially across successional gradients {van de Voorde et al., 2012 #1180}. Here, we examine

how chemical concentration and composition of the early succession plant species *Jacobaea vulgaris* Gaertn. (syn. *Secenio jacobaea* L., *Asteraceae*) varies with phenological development during the growing season and how this variation may depend on successional stage of the environment. We studied plant chemistry during three times of the year in early, mid and late stages of secondary succession using a well-established chronosequence of ex-arable fields.

Optimal defence theory assumes that there are costs involved in allocating resources to growth and defences (McKey, 1974; Rhoades, 1979). According to this theory, plants should optimize their defences to protect the most valuable plant parts. Flowers should thus be better defended than older leaves. Allocation of defences within a plant is therefore expected to change during a plants' life time and during the season. Plants are 'smart' investors (Van Dam et al., 1996) and herbivory can result in defence induction (Karban and Baldwin, 1997). Strong seasonal herbivore

pressure can induce plant defences (Haukioja, 1980; Shiojiri and Karban, 2008). Often, plant defence levels increase throughout the season (Brooks and Feeny, 2004; Iason et al., 2012). But even under controlled greenhouse conditions, secondary plant chemistry has been shown to change in plants grown at different times in the year (Gols et al., 2007). Besides defence compounds also nutrients and photosynthetic compounds vary with plant phenological development, depending on plant life history strategy. For example, Amsellem and Mckey (2006) showed two contrasting tree strategies: *Leonardoxa africana* delayed greening the leaves until full leaf expansion while *Barteria nigritana* photosynthesized during leaf expansion. Differences in the phenology of leaf development were then reflected in the duration of larval development of the main herbivores observed on these two plants and may have influenced its ant protection strategies. The strategy of phenological defence may thus affect the evolution of biotic defence. Relocation and/or shift of primary metabolite content, therefore, can be advantageous for plant fitness especially when seasonal relocation will favour those plant organs that are most in need of these compounds. However, attacks by herbivores can result in reduced rates of photosynthesis and change of carbon and nutrients balance (Bryant et al., 1983; Tuomi et al., 1984).

Although the composition and concentration of plant defence compounds may change throughout succession (Walters, 2011) few studies have addressed differences in plant defences along successional gradients{Rasmann et al., 2011 #1031}. Plants are exposed continuously to differing conditions of light, temperature etc., which promotes variations in primary and secondary chemistry both during the season and between different stages of vegetation succession (Gols et al., 2007; Tilman, 1987; Walters, 2011). The nitrogen and phosphorus content in the soil is known to shift along most chronosequences (Richardson et al., 2005). For example, in an Australian chronosequence nitrogen tends to limit plant growth in relatively young fields while ancient (>1000 years) fields tend to be phosphorous limited (Lambers et al., 2008). Plants are plastic in an array of traits, which helps to overcome nutrient limitations. These plastic responses include effective mechanisms of resorption, internal recycling, allocation and use of nitrogen and phosphorous in growth and defence (Lambers et al., 2008; Richardson et al., 2005; Walters, 2011). Mason et al. (2012) showed that in a chronosequence contrasting growth forms (angiosperms, conifers, tree ferns) all declined in leaf nitrogen and phosphorous concentrations. The declines mounted between 67% and 88% along the soil chronosequence. In addition to soil nutrient limitation plants face aboveground light competition as plant community composition shifts during vegetation succession and seasonal growth. Some studies have suggested that low nutrient and little disturbed habitats tend to have vegetation succession from light specialists to nutrient specialists, while vegetation in high nutrient or highly disturbed habitats evolve to light specialists. While many succession studies focus on differences between species (Rees and Bergelson, 1997; Tilman, 1982, 1987), populations of the same species may also vary in both exposure to environmental conditions (Van de Voorde et al., 2012) growing in fields of different along successional stages.

We studied how important the stage of vegetation succession is for the variation of primary and secondary metabolites between different populations of *J. vulgaris* throughout the growing season. *J. vulgaris* is a native biennial weed infamous for its production of pyrrolizidine alkaloids (Witte et al., 1992). For our study, we used a well-established chronosequence of ex-arable fields near Veluwe National Park in the Netherlands, where, management aiming at re-creating open grasslands with a high biodiversity has resulted in a series of abandoned fields that can be used to reconstruct the performance of *J. vulgaris* during time since abandonment at one moment in time (van de Voorde et al., 2012). These ex-arable fields are colonized by *J. vulgaris* according to a hump-shaped population development booming during the first 5–7 years and then busting during the next 10–20 years following land abandonment (van de Voorde et al., 2012). This pattern was attributed, at least to some extent, to level of control by soil biota and negative indirect feedback effects from other plant species that gradually colonize the old fields (Kostenko et al., 2012; van de Voorde et al., 2012).

An earlier garden experiment with *J. vulgaris* (Aplin and Rothschild, 1972) showed that pyrrolizidine alkaloids (PAs) in leaves reached highest concentrations in June with a steep decrease later in the season. De Boer (1999) found that nitrogen percentage and PAs tend to decrease with leaf age in the plant. Studies on close related species *Senecio vulgaris* and *Senecio vernalis* further showed that the highest PA concentrations is found in the flowers (Hartmann and Zimmer, 1986). Populations of *J. vulgaris* can differ in their PA composition (Macel et al., 2004; Witte et al., 1992). Under natural conditions temporal variation of nitrogen or chlorophyll in *J. vulgaris* is, as far as we are aware of, unknown. As the chronosequence we are using for this study is not nutrient limited we should expect that vegetation develops towards light competitive specialists (Rees and Bergelson, 1997; van de Voorde et al., 2012; van der Wal et al., 2006), thus *J. vulgaris* should shift towards higher chlorophyll contents.

We addressed the following questions: (i) how may field age (time since abandonment) affect plant chemistry, (ii) is the seasonal variation consistent between succession stages, and (iii) have *J. vulgaris* flowers in the field higher concentrations of pyrrolizidine alkaloids than leaves, and were these concentrations affected by succession stage? Our hypotheses were that: (a) there was a seasonal allocation of nutrients and defence metabolites to reproductive organs that fitted the optimal defence theory; (b) this seasonal/organ variation in chemistry would be dependent on the successional stage of the vegetation with an increase of defences and higher chlorophyll levels in later succession.

Results

Plant chemical concentrations

The factors season and succession stage had significant effects on leaf and flower chemical content, ($P < 0.05$; Tables 1 and 2), and these factors significantly interacted in the leaves chemical content and the PAs for flowers ($P < 0.002$, Tables 2 and 3). Flowers in Mid succession (10–20 years abandoned) fields had higher tertiary-amine levels than flowers in Young (0–10 years) or Old (20–30 years) fields, especially in early Summer (Fig. 1 or Table 2). In late Summer, PA N-oxide contents in flowers and leaves increased with time since abandonment, however that did not occur in Spring or early Summer (Fig. 1). In all fields, in early Summer flower heads had higher levels of PA tertiary-amines than leaves. In late Summer, however, the tertiary-amines and N-oxides contents in leaves were not significantly different from the flower heads (Fig. 1, Fig. S1). Nitrogen concentration in flowers was highest in late Summer independent of succession stage. Leaves in general had higher concentrations of chlorophyll a, nitrogen and PA tertiary-amines in Spring. In early Summer leaves had a higher content of chlorophyll b than in Spring or late Summer (Fig. 2). PA tertiary-amines in leaves showed higher levels in Spring when compared with the other seasons (Fig. 1). Leaves from Old fields had higher chlorophyll a content than leaves from the Medium or Young succession fields during Spring and early Summer but concentrations were the same in all fields in late Summer. Leaves from Young fields had the lowest chlorophyll b except for the late summer in the old field (Fig. 2).

Table 1
Effect of season, vegetation succession stage and field location on the concentrations of primary and secondary compounds of *J. vulgaris* leaves. PA- pyrrolizidine alkaloids; tertiary-amines and N-oxides are two different forms of PAs. Table entries are *F* values of ANOVA. PA data were log transformed.

Factors	df	Chlorophyll a	Chlorophyll b	Chlorophyll total	Nitrogen	Carbon	Total PA	PA T-amines	PA N-oxides
Season	2	27.385***	35.940***	41.699***	40.323***	9.002***	6.165**	17.456***	12.209***
Succession class	2	6.016**	7.357**	7.211**	5.023**	1.2	3.608	7.229**	1.816
Field (nested in succession)	5	1.706	2.459*	2.052	2.616*	13.618***	0.676	8.236***	1.699
Season* succession	10	3.325***	3.257**	3.674***	3.268***	5.551***	2.192*	2.316*	1.848*
Error	326	0.002	0.002	0.006	0.244	2.3	14.923	0.755	14.126

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 2
The effect of season, vegetation succession stage and field on the chemical concentration of primary and secondary compounds of *J. vulgaris* flowers. PA - pyrrolizidine alkaloids; tertiary-amines and N-oxides are two different types of PAs, all Log transformed. Table entries are *F* values of ANOVA.

Factors	df	Nitrogen	Carbon	Total PA	PA T-amines	PA N-oxides
Season	1	49.69***	2.67	253.01***	226.3***	176.37***
Succession	2	0.03	2.65	4.13*	6.46**	5.58**
Field (nested in succession)	3	3.53*	1.17	4.91**	7.59***	7.91***
Season* Succession	5	1.15	0.96	5.34***	8.19***	4.11***
Error	219	0.1	11.44	0.03	0.01	0.03

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 3
Selected ex-arable fields, their code, age, years of abandonment at the time of the study, and succession class. For plant community characteristics of the different fields see van de Voorde et al. (2011) and soil characteristics see Kardol et al. (2006).

Field	Code	Age (abandonment time)	Year of abandonment	Succession class	Latitude (N)	Longitude (°E)
Oud reemst	OR	5	2005	Young	52.02	5.48
Reyerskamp	R	5	2005	Young	52.01	5.47
Telefoonweg	T	7	2002	Young	52.00	5.45
Assel	A	7	2002	Young	52.12	5.49
Mossel	M	15	1995	Medium	52.03	5.45
Nieuw Reemst	NR	20	1990	Medium	52.04	5.47
Wolfheze	W	22	1988	Old	51.60	5.47
Dennenkamp	D	27	1982	Old	52.02	5.48

Comparison of PA composition between fields and season

Although small, the redundancy analyses (RDAs) revealed an effect of season and succession stage on the PA composition of both leaves and flowers. In leaves 6.7% of the variation was explained by season ($P = 0.002$), whereas succession stage explained 4.2% of the variation ($P = 0.042$). The PCA analyses for leaves showed that in early and late Summer leaves from Mid succession fields tended to be characterized by the lack of hydroxyjacobine (HOJb) and hydroxyjacoline (HOJl), and the enhanced abundance of jacobine-type PAs. In Young fields and in early season leaves were generally characterized by highest levels of acetylerucifoline (AcEr) and erucifoline (Er) (Fig. 3, Table S2).

In flowers 15.6% of the variation of PA composition was explained by season ($P = 0.001$) and 4.8% by succession stage, ($P = 0.007$) (Fig. 4). In late Summer, flowers had high levels of AcEr, Er, their N-oxides and senecionine (Sn) in all fields. In early Summer, Young fields had less common PAs, such as dehydroerucifolorine (DHEf) and its N-oxide (DHEf-ox) were more abundant. Flowers in Medium succession fields in late summer had high levels of jacobine (Jb), jacoline (Jl) and jaconine (Jn) and their corresponding N-oxides and hydroxy-PA metabolites, while Old and young fields were similar in composition when in late summer season (Fig. 4, Table S2).

Comparison of PA composition in leaves and flowers

In both seasons, PA composition differed between leaves and flowers (Fig. 5, Fig. S2). RDA analysis showed that more than 25% of the variation was explained by the effect of organ ($P = 0.001$). Leaves correlated with higher concentrations of jacobine (Jb) and its N-oxide (Jb-ox), jacoline N-oxide (Jl-ox), and lower concentrations of erucifoline N-oxide (Er-ox) concentration compared to flowers. The general differences in PA composition between the flowers and in the leaves remained the same throughout the seasons and successional stage.

Discussion

In this study we analysed the temporal variation of chemistry of leaves and flowers of *J. vulgaris* in relation to the stage of secondary succession. As expected, we observed that chemical concentrations in leaves and flowers depended on season. More interesting is our finding that the temporal variations of defensive chemicals were influenced, to some extent, by the succession stage. The interaction between season and successional stage resulted in a complex pattern of difference in plant chemical defences. Our data also suggest re-allocation of nitrogen from leaves to flowers from early to late Summer. Seasonal variation of nitrogen concentration, being high-
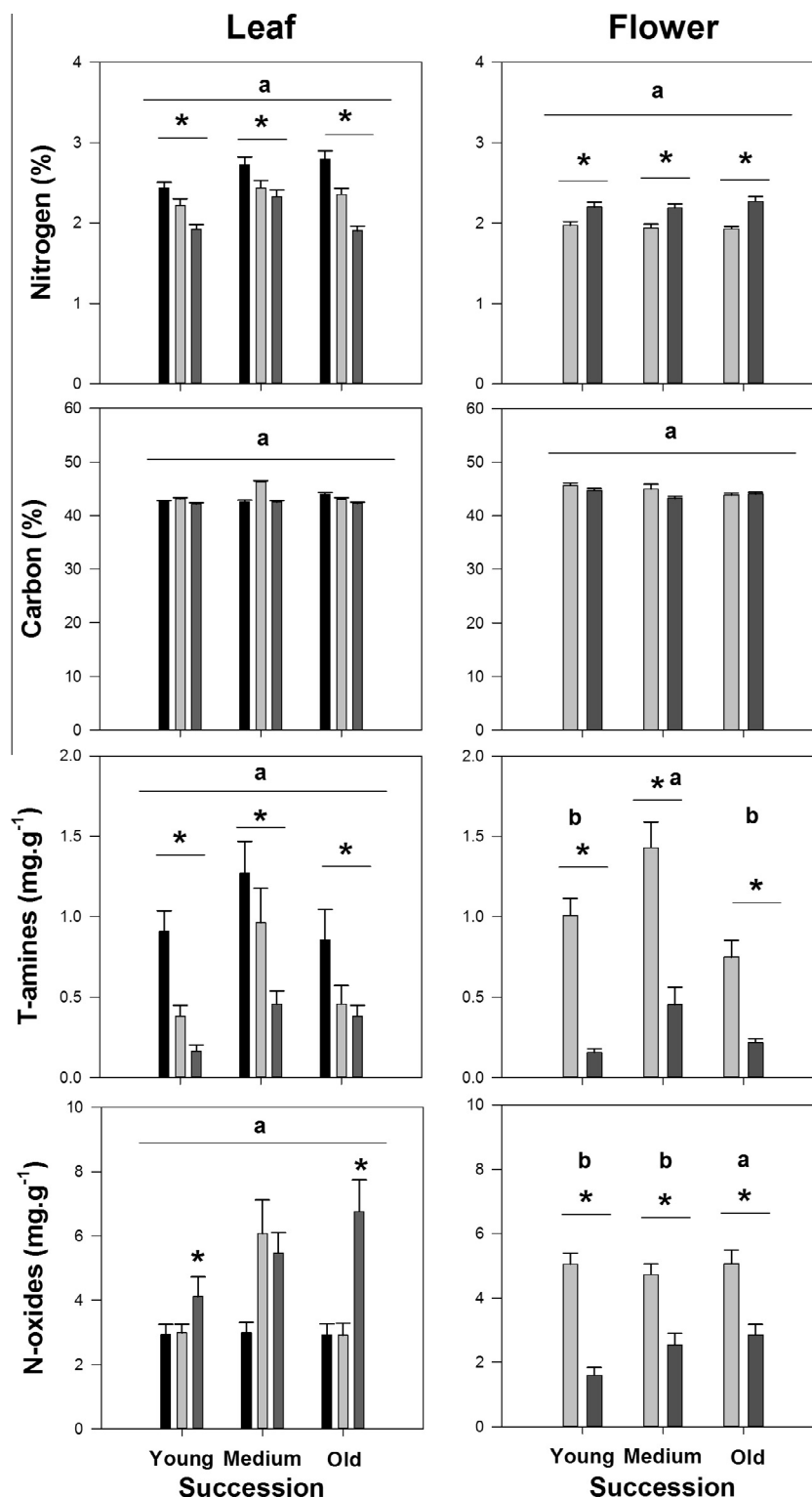
Fig. 1. Chemical content in leaves (left column) and flowers (right column) of *J. vulgaris* by field succession stage. Bars represent seasons, black-Spring; light grey-early Summer, dark grey-late Summer. Star symbol (*) represent significant differences between seasons (ANOVA <leaf *P* < 0.005, flower *P* < 0.001), letters represent significant differences between succession classes (ANOVA, leaf *P* < 0.05, flower *P* < 0.005). Error bars are standard errors.

est in leaves in spring and increasing in flowers at the end of summer, was consistent with other studies on the related species *S. vulgaris* (Qasem and Hill, 1995), but also with grasses (Jaeger et al., 1999). Jaeger et al. (1999) related the seasonal decrease of nitrogen in grasses to inability of the soil microbial community to sequester soil nitrogen in Spring, while in later seasons soil microbes could limit the plants access to nitrogen. A similar soil

process could be occurring in *J. vulgaris*, as in the chronosequence nutrient mineralization by the soil food web has been shown to vary during the growing season (Holtkamp et al., 2011). However, most likely the decrease of nitrogen content in the leaves is due to its re-allocation to flower heads, which shows an increase throughout the growing season. Chlorophyll concentration was lowest in late Summer, which is to be expected as *J. vulgaris* is a monocarpic
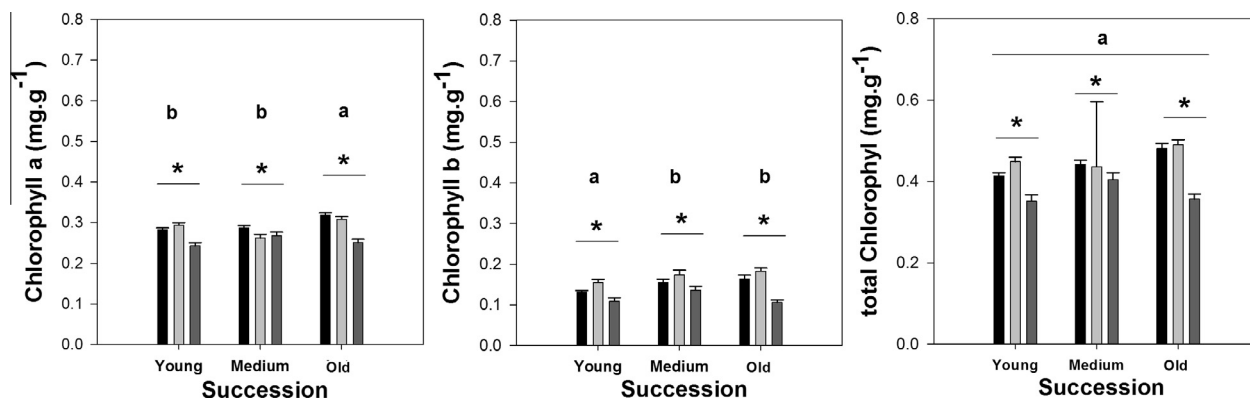
Fig. 2. Mean chlorophyll content in leaves of *J. vulgaris* by field succession stage. Bars represent seasons, black-Spring; light grey-early Summer, dark grey-late Summer. * represents significant differences between seasons (ANOVA, $P < 0.001$), letters represent significant differences between field succession stages (ANOVA, $P < 0.005$). Error bars are standard errors.
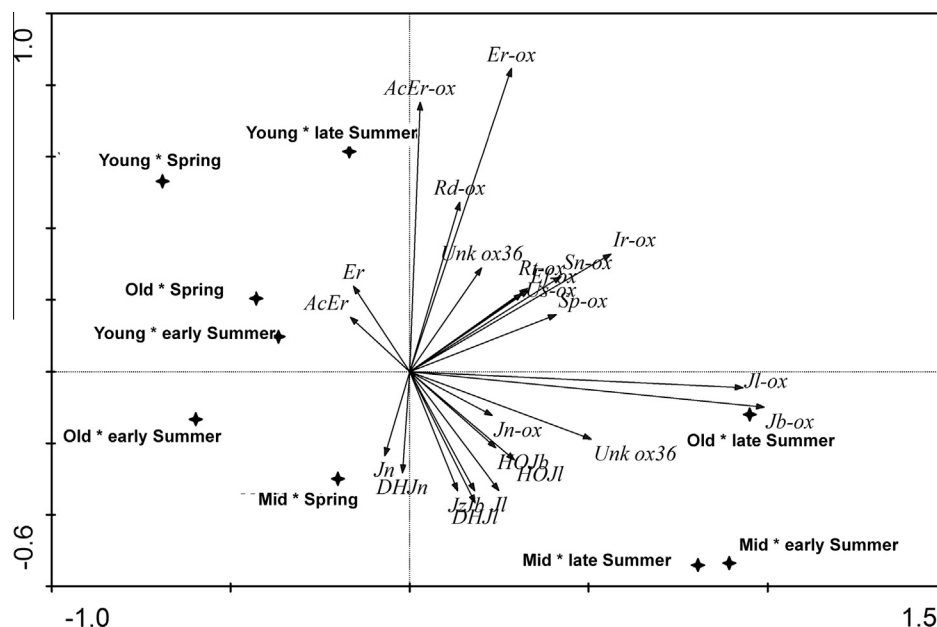

Fig. 3. Principal component analysis (PCA) of the pyrrolizidine alkaloid (PA) composition in leaves of *J. vulgaris*. PCA was done on log-transformed concentrations with succession class and season interaction. Axis 1 explains 48.8% of the variation, axis 2 explains 19.9%. Redundancy analysis showed 6.7% of the variation was significantly explained by the effect of season ($P = 0.002$) and 4.8% by succession class ($P = 0.007$). Star symbols represent age category*seasons group position. Arrows represent the different PAs (inclusion rule 20%; see Table S1 for list of PA names).
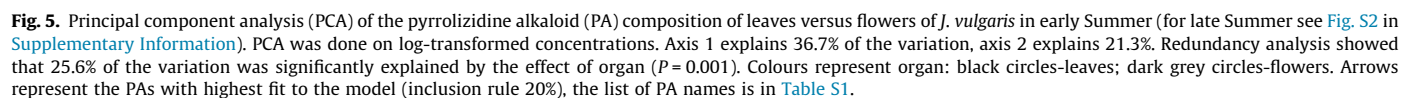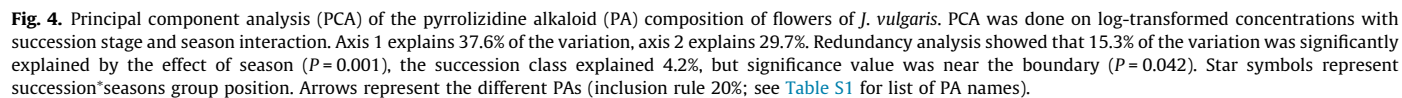
biennial species that dies after seed production. Towards the end of the life cycle plants senesce and high chlorophyll levels will drop. Other studies have shown that chlorophyll variation in plants depends on field site and chlorophyll amounts being generally lower late in the growing season (Joiner et al., 2011; Zhao et al., 2005).

Chlorophyll a was highest in the oldest fields and chlorophyll b was higher in the medium and old fields. It is known that vegetation structure and composition creates differences in plant competition for light (Tilman, 1988). With increasing time since abandonment the vegetation structure becomes more complex and vegetation denser (Kardol et al., 2005). The ground cover of *J. vulgaris* also decreases with field age (van de Voorde et al., 2012). The higher contents of chlorophyll a in older fields suggest increased light competition with surrounding vegetation when time since abandonment increases.

Earlier studies have shown that PA content can be affected by both aboveground and belowground biota (Joosten et al., 2009; Macel and Klinkhamer, 2010; Witte et al., 1992). These

aboveground and belowground biota are known to change throughout secondary succession (van de Voorde et al., 2011), which can contribute to variation in plant chemistry among successional stages. In flowers PA concentrations were higher in early than in late Summer. This coincides with the peak of herbivory which is in early summer in this climate zone (de Boer, 1999; Van der Meijden et al., 1989). A factor that could play a role here is that during the ripening of the flowers in late Summer nutrient flux is reduced and the influx of new PAs as well.

When analysing PA composition it was confirmed that jacobine was the most abundant PA in this plant species. High levels of jacobine are associated with plant toxicity towards generalist herbivores, but also with a lower fungal diversity in the rhizosphere (Kowalchuk et al., 2006; Leiss et al., 2009). Yet, jacobine is also known for having a positive effect on the feeding patterns of the specialist herbivores (Macel and Klinkhamer, 2010). Acetylerucifoline has been associated with soil legacy effects from past plant herbivory history (Kostenko et al., 2012). A number of PAs were

Fig. 4. Principal component analysis (PCA) of the pyrrolizidine alkaloid (PA) composition of flowers of *J. vulgaris*. PCA was done on log-transformed concentrations with succession stage and season interaction. Axis 1 explains 37.6% of the variation, axis 2 explains 29.7%. Redundancy analysis showed that 15.3% of the variation was significantly explained by the effect of season ($P = 0.001$), the succession class explained 4.2%, but significance value was near the boundary ($P = 0.042$). Star symbols represent succession*seasons group position. Arrows represent the different PAs (inclusion rule 20%; see Table S1 for list of PA names).

Fig. 5. Principal component analysis (PCA) of the pyrrolizidine alkaloid (PA) composition of leaves versus flowers of *J. vulgaris* in early Summer (for late Summer see Fig. S2 in Supplementary Information). PCA was done on log-transformed concentrations. Axis 1 explains 36.7% of the variation, axis 2 explains 21.3%. Redundancy analysis showed that 25.6% of the variation was significantly explained by the effect of organ ($P = 0.001$). Colours represent organ: black circles-leaves; dark grey circles-flowers. Arrows represent the PAs with highest fit to the model (inclusion rule 20%), the list of PA names is in Table S1.

more abundant in *J. vulgaris* flower heads than in leaves, such as senecionine, integerrimine, seneciphyline and their N-oxides. Although the concentrations of senecionine, integerrimine and seneciphyline are lower when compared to erucifoline or jacobine, their presence in the flower heads suggests that these PAs have a role in flower protection (Hartley et al., 2012). Senecionine and seneciphylline are deterrents of generalist insect herbivores (Dreyer et al., 1985; Macel et al., 2005) and integerrimine is an effective fungal inhibitor (Hol and Van Veen, 2002). Interestingly, the flower PA composition could be attractive to the specialist herbivore *Tyria jacobaea* thus enhancing oviposition (Macel and Vrieling, 2003). The differences in PA composition of leaves and flowers suggest different roles of PAs towards damage by herbivores and pathogens. Defence of flowers has a trade-off, as mutualists and pollinators of the plant should not be deterred whilst preventing herbivory (Irwin et al., 2004).

Our study does not distinguish whether the effects in plant chemistry are solely due to seasonal (phenological) and succession factors or if the genetic variation among plants may play a role as well. Succession and season interactions explained only a limited percentage of the variation. PA profiles and concentrations are at least partly genetically determined in *J. vulgaris* (Macel et al., 2004; Vrieling et al., 1993). Since PA profiles alter with soil biota (Carvalho et al., 2012; Joosten et al., 2009) these chemical changes could be partly attributed to phenotypic plasticity. To distinguish between genetic differentiation (selection on particular genotypes) and phenotypic plasticity (changes induced by the environment) further studies are needed. In conclusion, both concentration and composition of chemicals in *J. vulgaris* varied throughout the growing season, and several chemicals were affected by successional stage as well. Thus far, the role of succession in changes of chemical composition of a species has received little attention, but it may provide an interesting frame for studying the role of both ecology and evolution in shaping differentiation of plant chemistry.

Experimental

Species description

J. vulgaris Gaertn (syn. *S. jacobaea* L., Asteraceae) is a monocarpic biennial to short-lived perennial. In the first year this species forms a rosette, whereas flowering occurs in the second year if conditions are favourable (Harper and Wood, 1957; Wesselingh and Klinkhamer, 1996). If not, then flowering can be postponed for one or more years (Van der Meijden and van der Waals-Kooi, 1979). Although native to the Netherlands, *J. vulgaris* is considered a noxious weed, as it is a pioneer species that can become highly dominant in arable fields, unless it is controlled by mechanical or chemical means (van de Voorde et al., 2011). This species contains pyrrolizidine alkaloids (PAs) that have hepatotoxic as well as carcinogenic properties towards many herbivores, including livestock (Macel, 2011; Mattocks, 1986). The plant species has also antifungal activity (Hol and Van Veen, 2002) that can affect, and is affected by, soil biota and insects (Joosten et al., 2009; Macel et al., 2005). Toxicity of *J. vulgaris* is largely due to its PA content. More than 30 different PAs can be found in this species in all plant organ types, from roots, stems and leaves to flower heads and seeds (Mattocks, 1986, Table S2). *J. vulgaris* populations can differ in their PA concentration in flowers (Hartmann and Witte, 1995) and leaves (Macel et al., 2004).

Field selection

We selected 8 fields (Table 3) located at south Veluwe, the Netherlands, where agricultural production had ceased between 5 and 30 years ago. Currently, these fields are being subjected to nature restoration (Bezemer et al., 2006). *J. vulgaris* density in these fields increases in the first five to seven years of land abandonment and then declines according to a hump-shaped pattern (van de Voorde et al., 2012). This typical population dynamics is attributed, at least to some extent, to plant–soil interactions (Bezemer et al., 2006; van de Voorde et al., 2012). Based on previous findings of negative, neutral, and positive plant–soil feedback in early-, mid- and late-successional fields (Kardol et al., 2006) we grouped fields in successional age classes: Young (0–10 years of abandonment), Medium (10–20) and Late (20–30).

Sample collection

In each field we followed a sampling scheme similar to van de Voorde et al. (2011) by establishing an imaginary W-shaped transect that covered the whole field. Every 5 m a plant sample was collected resulting in a total of 20 plant samples per field. As the fields were not similar in size, the samples were collected from the centre of the fields in an area of 30 × 100 m. Sampling was carried out three times during the growing season: the rosette stage (Spring), the flowering stage (early Summer) and the senescing/ seed stage (late Summer). In the sampling year (2010) the Spring rosette was sampled in May, flowering took place in June, and senescence occurred in late August. In early and late Summer the flowers were collected from the same plant as the leaves, but each season different plants were randomly sampled. In total 8 fields * 3 seasons were planned to be sampled. However, two fields (Telefoonweg and Assel; see Table 3) were mown before flowering stage, so that it was impossible to collect plant samples from these two fields during early and late Summer.

Chemical analysis

From each individual plant we collected five leaves from basis to top of the shoot in order to include leaves of various ages. We also collected four flower heads when plants were flowering. Leaves and flowers were analysed separately for chlorophyll a and b (mg/g), nitrogen (% of dry weight - dw), carbon (% dw) and pyrrolizidine alkaloids (mg/g dw). We did not measure chlorophyll content of flowers. Per individual plant the 5 leaves or the 4 flowers were pooled together to extract an average organ chemical content. For the chlorophyll extraction one 10-mm diameter disc was collected from each leaf. The discs were cut out, immersed immediately in 3 mL of dimethyl sulfoxide (DMSO) and stored in a dark room for three days at constant room temperature. In a spectrophotometer (Genesys 20 spectrophotometer 4001/4, Thermo Fisher Scientific, Waltham, MA, USA) absorbance (Abs) at 649 nm and 665 nm was measured and chlorophyll concentrations (initially in μg/mL) were calculated using the equation:

$$Chla = 12.19 \times Abs(665\ nm) - 3.45 \times Abs(649\ nm)$$

$$Chlb = 21.99 \times Abs(649\ nm) - 5.32 \times Abs(665\ nm)$$

The leaves were freeze-dried for 96 h after chlorophyll analysis. Thereafter the leaf samples were homogenized and fast-ground to a fine powder for PA analysis and Nitrogen (N) and Carbon (C) analyses. For the C:N analysis 6 mm diameter metal cups were selected and 3–5 mg of dried powder used. Combustion–reduction was done in a C:N analyser (Thermo flash EA 1112, Thermo Fisher Scientific, Waltham, MA, USA) for C:N percentage estimation.

Pyrrolizidine alkaloids were extracted according to Joosten et al. (2011). We extracted 10 mg of powdered material with 1 ml of 2% formic acid solution containing heliotrine as internal standard (1 μg/ml). The extract was then filtered and 25 μL were diluted 40 times with 10 mM ammonium hydroxide. The PA content was

determined by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a Waters Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Waters Premier XE tandem mass spectrometer (Waters, Milford, MA, USA). Separation was achieved on a Waters C18 BEH column (150×2.1 mm, 1.7 μm particles) using 5 mM ammonium hydroxide as mobile phase and acetonitrile as organic modifier (0–50%) in a 12-min linear gradient. The mass spectrometer was operated in positive electrospray mode and the samples were screened for a total of 45 PAs. Details on the mass spectrometric conditions can be found in Table S1. PAs were quantified against a set of PA standards added to *Tanacetum vulgare* plant extract (which itself is free of PAs) to minimize matrix effects that otherwise could play a role when using standards in solvent only. The calibrant solution was injected every 25 samples to check for variations in detector response. Samples were injected in a randomized order. Data were processed using Masslynx 4.1 software (Waters, Milford, MA, USA). For a number of PAs no reference standard was available. For these compounds quantification was performed by comparison with a structurally related compound, indicated in Table S1. Data on individual compounds were summed to obtain total PA, tertiary-amine and N-oxide content and the concentration of the main PA types present in *J. vulgaris*.

Statistical analysis

The effects of vegetation succession class (Table 3) and season on chemical content of leaves and flowers were examined by analysis of variance (ANOVA). Succession class and season were added in the model as fixed factors. To accurately test the effect of succession the fields were nested in the factor successional class as a random term. Differences between groups were analysed with Post-hoc Tukey HSD tests. To meet the assumptions of normality and homoscedasticity total pyrrolizidine alkaloids, tertiary-amines and N-oxides were log-transformed. The ANOVA analyses were performed in SPSS 17.0 for Windows.

Since PAs in *J. vulgaris* are highly diverse, leaf and flower PA composition were analysed with multivariate statistics to study changes in composition. The most appropriate multivariate analyses were chosen by detrended correspondence analysis (DCA). With DCA we tested for linear (principal component analyses – PCA, and redundancy analyses – RDA) or Unimodal (correspondence analyses – CA co-correspondence analyses – CCA). As all gradients were smaller than 3, linear analysis was chosen (Lepš and Šmilauer, 2003). The explanatory power of season, field origin and succession class for the variation in the PA composition was evaluated by RDA. Monte Carlo permutations (999 permutations) were used to test the significance of all axes. All multivariate analyses were performed in CANOCO 4.5 for Windows.

The differences between plant organs (leaf vs. flower) were analysed per season. In each season we examined the chemical concentration differences between flowers and leaves by analysis of variance (ANOVA). Field and plant organ were fixed factors. PCA and RDA were applied to examine the chemical composition differences of the organs within each season.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.12.004.

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